AFFINITY-CONTROLLING MATERIAL WITH THE USE OF STIMULUS-RESPONSIVE POLYMER AND SEPARATION/PURIFICAITON METHOD WITH THE USE OF THE MATERIAL

Technical Field

This invention relates to an affinity-controlling material comprising (a) a stimulus-responsive polymer and (b) an affinitive substance (ligand) having affinity for a target substance that is present in a solution in contact with the material. By subjecting the material to a stimulus, for instance a physical stimulus, the chemical and/or physical environment provided around the ligand can be changed thereby changing the affinity between the ligand and the target substance. The change in environment is typically related to a conformational change in the stimulus-responsive polymer.

The term "solution" as used herein means liquids, typically containing dissolved buffer components and salts (ions). Typical solutions have as the liquid component: a solvent such as water, an organic solvent and mixtures thereof. Organic solvents include in particular those that are miscible with water, for example, water-miscible alcohols, acetonitrile, tetrahydrofuran, etc and mixtures thereof. The terms "change in affinity", "changing the affinity" etc refer to the apparent affinity, i.e. the affinity that can be measured under the conditions applied.

Affinity-controlling material will further on also be called separation medium or separation material.

The pres nt invention further relates to an affinity-

controlling material with which a desired target substance (metal ions, drugs, biological components, etc.) can be removed or separated and/or purified from mixtures containing other substances.

The present invention furthermore relates to a method for separating and purifying target substances (metal ions, drugs, biological components, etc.) with the use of the above-mentioned affinity-controlling material. The method preferably comprises keeping at least one condition other than temperature (for example, pH value of solution, organic solvent concentration or salt concentration) constant.

Background Art

There have previously been employed ion exchange chromatography, reversed phase chromatography, and other affinity chromatography principles and various batch-wise protocols based on affinity binding as means for efficaciously separating and purifying biological components, drugs, etc from mixtures of substances. With the recent advances in biotechnology, a number of novel physiologically and biologically active substances including recombinant proteins have been developed. At the same time there has been an increased need for improved methods for separating and purifying these substances without unacceptable losses in biological and physiological activity.

Separation and/or purification of a target substance from a mixture of substances by affinity chromatography and oth r adsorption bas d separation techniques typically encompass a

binding step (adsorption step) and a release step (desorption or eluting step). Compared to the binding step, the release step typically requires a change in the composition of the liquid in contact with the separation medium. Illustrative examples for accomplishing the appropriate change are adding an organic solvent to a mobile phase, elevating the salt concentration of the mobile phase, or changing the pH value of the mobile phase. This also applies to the turbulent or non-turbulent liquid phases used in batch-wise procedures. These operations result in an increased risk for inactivation of physiologically active substances. Even if an active substance often may be separated and purified by conventional chromatographic techniques without unacceptable losses in activity, the organic solvent, salt, etc added to the mobile/liquid phase should in most cases at least be partially removed from a purified or isolated target substance. This leads to an additional risk for lowering the activity and/or recovery/yield of the target substance.

During the last decade there has been an interest in combining so called stimulus-responsive polymers with chromatographic techniques and other techniques based on binding or partition of a desired substance to an insoluble separation medium of the type used in chromatography.

Recently, separation media comprising ion exchanging groups that are covalently attached to stimulus-responsive polymers have been described. See for instance JP application 140722/98 with corresponding patent application WO 99/61904

Galaev et al (J. Chromatog. A 684 (1994) 37-43 and WO 94/154951 describe temperature elution of a target substance in a chromatographic system in which a plurality of ligand groups

is covalently attached to a base matrix. A temperature responsive polymer is indirectly affinity bound to the ligand groups by multi-point attachment, i.e. the attachment of the thermo-responsive polymer is depending on the prior attachment of the ligands to the base matrix. By changing the temperature the ligand becomes more or less prone to affinity bind to its target substance.

Ohnishi et al (JP-A-09 049830), Ohnishi (JP-A-08 103653), Ohnishi (JP-A-07 136505) and Ohnishi (JP-A-07 135957) disclose separating materials comprising a stimulus-responsive polymer and a substance having specific affinity on the surface of a support matrix. However, these documents only report the elution of a target substance by a change in temperature based on a separation system in which a copolymer (complex) obtained by coupling a ligand to a temperature-responsive polymer is attached to a base matrix. When such an complex is used, because the elution temperature of a target substance varies depending on the kind or nature of a ligand, temperature control must be performed by changing the kind of a complex according to the kind of a ligand.

Hofman et al., (WO 8706152) describe a separation method in which the ligand is attached to a temperature responsive polymer. Binding and elution of the target substance occur at the same side of the critical solution temperature. For the term critical solution temperature se further under the discussion about thermo-responsive polymers.

There are also a number of publications describing chromatography based on separation material comprising stimulus-responsive polymers but without having a ligand

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covalently attached to the temperature-responsive polymer. Gewehr et al (Macromolecular Chemistry and Physics 193 (1992) 249-256) describe gel chromatography on porous silica beads coated with a temperature-responsive polymer. Hosoya et al (Anal. Chem. 67 (1995) 1907-1911); Yamamoto et al. (Proc. 114[th] National Meeting of the Pharmaceutical Society of Japan, Tokyo (1994) 160; Kanazawa et al (Yakugaku Zasshi 117 (10-11) (1997) 817-824; Kanazawa et al (Anal. Chem. 68(1) (1996) 100-105); Kanazawa et al (Anal. Chem. 69(5) (1997) 823-830); Kanazawa et al (J. Pharm. Biomed. Anal. 15 (1997) 1545-1550); Yakushiji et al (Langmuir 14(16) 1998) 4657-466268); Kanazawa et al (Trends Anal. Biochem. 17(7) (1998) 435-440); Yakushiji et al (Anal. Chem. 71(6) 1999) 1125-1130); Grace & Co (EP 534016); Okano (JP 6-108643) describe

reversed phase chromatography on matrices covered by a thermoresponsive polymer for the separation of biomolecules. The matrices may be porous. The hydrophobic groups utiliz d are inherent in the polymer as such. There is no ligand that has been covalently attached to the polymer after polymerisation.

Certain aspects of the general ideas of performing separations on chromatography on (a) a separation medium covalently functionalized with a conjugate between a stimulus-responsive polymer and an affinity ligand and (b) a separation medium functionalized by separate/independent attachment of a stimulus-responsive polymer and an affinity ligand were presented at National meetings of Chemical Society of Japan on March 28, 1999 and on May 27 1999 (SPSJ, the Society of Polymer Science, Japan, Annual Meeting, Abstract p583) respectively.

Separation media are often in the form of particles that may be porous and non-porous. The particles typically comprise a base matrix to which the ligand is attached directly or indirectly, for instance via a spacer. The particle material may be a synthetic polymer such as crosslinked polymerisates of styrenes, acrylates/methacrylates and the like. However, polystyrene particles and polymethacrylate particles per se are relatively hydrophobic and therefore often exhibit a pronounced non-specific adsorption of various substances that may be present together with a desired target substance. When such particles are to be used as a support in affinity-based separations, it is therefore necessary to make th m sufficiently hydrophilic in order to minimize the hydrophobic/non-specific adsorption. To improve the separation and purification

performance of particles, it is often favorable to use particles of uniform size (monosized or monodispersed particles). Although there has been a demand for hydrophilic synthetic polymeric supports in form of porous particles of uniform size for a long time, there have been known few methods for producing the same in practice.

Disclosure of Invention

The objects of the invention are to provide solutions to the problems discussed above thereby enabling improved separation methods and separation materials.

The present inventors have conducted intensive studies and found that an affinity-controlling material/separation medium can be synthesized by attaching a stimulus-responsive polymer and an affinitive substance (ligand) of a target substance independently to a support, i.e. via separate linking structures. The present inventors have further found that a target substance adsorbed by the above-mentioned affinity-controlling material can be desorbed under a physical stimulus, such as a temperature change, while keeping at least one conditions other than the stimulus concerned constant. If, for instance, the stimulus is a temperature change, the condition(s) to be kept constant may be selected amongst, for example, pH, organic solvent concentration, salt concentration etc. The present inventors have furthermore found that the bonding ability between a ligand and a target substance depends on the length of a spacer by which the ligand is attached to a support/base matrix or on the size of the stimulus-responsive polymer bond d to the support.

The pres nt inventors have also conceived to use hydrophilic porous polymer particles that may be of uniform size (monosized = monodispersed) as support material in the invention. In this part of the invention the inventors thus have conceived to use particles obtained by polymerisation of monomer emulsions/suspensions obtained by the membrane emulsification method. This embodiment also includes to chemically treat the particles with acidic or basic substances in order to introduce hydrophilic groups and/or groups that will enable covalent attachment of the ligand and/or the stimulus-responsive polymer, for instance hydroxy and/or amino groups. The chemical treatment requires that the starting material (monomer) used in the membrane emulsification method exhibits a reactive group that is able to react with the acidic or basic substance utilized. Typical reactive groups have been epoxy groups. The present invention has been completed based on these findings.

Accordingly, the present invention relates to an affinity-controlling material/separation medium of the type defined in the introductory part. One of the major characteristic features is that the stimulus-responsive polymer and the ligand are attached by separate/independent links to a base matrix. When changing the level and/or intensity of the appropriate stimulus for the polymer from one side of the critical level/intensity to the other side, there is caused a reversible change in the affinity between the ligand and its target substance. The stimulus may be a physical stimulus and is in the experimental part typified by a temperature change.

The pres nt invention further relates to an affinity-controlling material wherein the affinity of an affinative

substance of a target substance is reversibly changed by changing the chemical or physical environment of a stimulus-responsive polymer under a physical stimulus while keeping at least one condition other than the changed condition constant, i.e. subjecting the thermo-responsive polymer to a change in one physical stimulus while keeping at least one of the other stimulus constant. If the changed stimulus is the temperature, then at least one condition/stimulus except the temperature is maintained essentially constant (for example, pH, organic solvent concentration or salt concentration).

The present invention furthermore relates to an affinity-controlling material/separation medium as defined above wherein the affinity between the ligand and the target substance depends on

(a) the length of the spacer attaching the ligand to the base matrix or

(b) the size, for instance as reflected in molecular weight, of the stimulus-responsive polymer.

The present invention further relates to an affinity-controlling material/a separation medium as defined above wherein the support (base matrix) comprises hydrophilic porous polymer particles preferably having a uniform particle size and/or being produced by polymerisation of a monomer emulsion/suspension obtained from the membrane emulsification method. Hydrophobic particles exhibiting functional groups that are reactive with acidic or basic substances, may be rendered less hydrophobic by reaction with this kind of substances. Typical examples of reactive groups are epoxy groups, i.e. if the membrane emulsification method involves polymerisation the

starting monomer contains an epoxy group.

The present invention further relates to utilization of the above-mentioned affinity-controlling materials as a chromatographic packing.

An additional embodiment of the invention is a method for the separation of one or more target substances from a liquid sample (solution) (liquid I). This embodiment comprises the steps of

(a) bringing a liquid sample (liquid (I)) containing a target substance in contact with a separation medium (including a chromatographic packing) which is functionalized with a ligand which is capable of affinity binding to the target substance, said contact being under conditions permitting binding of said target substance to said ligand;

(b) contacting said carrier with a liquid (II) not containing said target substance under conditions such that the target substance is released from said ligand to liquid (II).

Between steps (a) and (b) the liquid sample is preferably separated from the separation medium. After step (b), liquid (II) may be separated from the separation medium. The target substance may, if so desired, be worked up from liquid II. The separation medium may be washed between step (a) and step (b).

The liquids typically have been aqueous for target substances that are biologically and/or physiologically active molecules, e.g. bioorganic molecules having structures selected amongst nucleotide structure (including nucleic acids), polypeptide structure (including proteins), carbohydrate structure, steroid structure etc.

This embodiment of the invention is characterized in that

(i) said separation medium comprises a support/base matrix to which a stimulus-responsive polymer as defined elsewhere in this specification and the ligand are linked separately, and

(ii) subjecting in step (a) and at least during binding of the target substance to the ligand, the separation media to a stimulus at a level/intensity at which the stimulus-responsive polymer is in a conformation enhancing binding of the target substance to the ligand, and

(iii) subjecting in step (b) and at least during release of the target substance from the ligand, the separatory material to a stimulus at a level/intensity at which the stimulus-responsive polymer is in a conformation hindering binding of the target substance to the ligand.

Preferably the same kind of stimulus is referred to steps (a) and (b). Compared to step (a), the level/intensity of the stimulus in step (b) is on the opposite side of the critical level/intensity for the stimulus-sensitive polymer used. The process can be made cyclic in case step a is repeated after step b, typically after separate washing/regeneration steps and equilibration steps.

Various embodiments of the inventive method may be carried out in a batch-wise or a chromatographic mode. Chromatographic modes, for instance, may be carried out by permitting the various liquids in plug flow (mobile phase) to pass through a bed of the separation medium while subjecting the bed to the appropriate stimulus for the individual steps and the stimulus-responsive polymer that is attach d to the base matrix. The bed may be a

porous monolith or a bed of packed or fluidised particles. Batch-wise modes concern suspended particles in combination with turbulent flow and/or turbulent liquids.

Brief Description of Drawings

Fig. 1 provides a chromatogram showing control of the affinity of BSA by BC-10 with temperature change.

Best Mode for Carrying Out the Invention

Stimulus-responsive polymer

The physical stimulus to be used in the present invention is exemplified by temperature.

Depending on the particular stimulus-responsive polymer used other stimulus may apply, for instance, light, magnetic field, electrical field, pH etc. Stimulus-responsive polymers are often called "intelligent polymers".

Stimulus-responsive polymers are characterized in that they upon being subjected to the correct kind and intensity or level of a stimulus undergo a conformational and reversible change of their physico-chemical properties. The change may be a switch from a pronounced hydrophobicity to a pronounced hydrophilicity or vice versa. The exact level/intensity the required stimulus at which the switch occurs is called critical level or critical intensity of the stimulus and will depend on the structure of the polymer and often also on other conditions (solvent, solutes such as salts etc). The most wellknown and most utilized polymers of this kind respond to heat (thermo-responsive or temp rature-responsive polym rs). Temp rature-responsive polymers are recognized by a sharp temp rature limit

at which they switch from a pronounced hydrophilic state to a pronounced hydrophobic state and vice versa. For temperature-responsive polymer in solution the change in conformation/physico-chemical properties occurs at the so-called critical solution temperature (CST).

For a temperature responsive polymer in aqueous media there is a lower critical solution temperature (LCST) or an upper critical solution temperature (UCST). For a polymer having a LCST, the polymer changes from a hydrophilic conformation to a hydrophobic conformation when the temperature is passing the LCST from below. For a polymer having an UCST, the change is the opposite when the temperature is passing the UCST from below. The exact value of the LCST and UCST depend on the polymer and also on other conditions applied (solvent, other solutes etc).

As discussed above one of the characteristic features of the invention when a temperature-sensitive polymer is used is that the binding to and the release from the ligand are performed at opposite sides of an applicable CST.

The stimulus-responsive polymer to be used in the invention preferably has an insignificant affinity for the target substance compared to the affinity between the target substance and the ligand attached to the support. Preferably there is no significant affinity between the ligand and the stimulus-responsive polymer.

Examples of the stimulus-responsive polymer to be used in the present invention include poly(N-substituted acrylamide) such as poly(N-isopropyl acrylamide), poly(N-substituted methacrylamide) such as poly(N-isopropyl methacrylamide), poly(N,N-disubstituted acrylamid), poly(N,N-disubstituted

methacrylamid), polymethyl vinyl ether, poly(ethyl ne oxide-propylene oxide) copolymer, polyvinyl alcohol derivatives typified by partly saponified polyvinyl alcohol and cellulose derivatives typified by methyl cellulose. It is also possible to introduce reacting functional groups (for example, amino, carboxyl or hydroxyl groups) into the stimulus-responsive polymer so as to covalently attach the stimulus-responsive polymer to the support/base matrix.

Ligands

Ligands may be attached to the base matrix via affinity bonds or via covalent bonds, preferably the latter. According to the present inventive concept it is not via the stimulus-responsive polymer.

One typical kind of ligands affinity binds to the target substance by more or less pure ionic (electrostatic) interactions. Alternatively the binding includes more complex interactions such as in conventional affinity binding (affinity adsorption). For ionic interactions, the ligands comprise positively or negatively charged entities (ion exchange; the immobilised entity being selected among primary, secondary, tertiary and quaternary ammonium, sulphonate, sulphate, phosphonate, phosphate, carboxy etc groups). More complex interactions are illustrated by the ligand being an individual affinity member in the pairs,

(a) antibodies and antigens/haptens,

(b) lectins and carbohydrate structures,

(c) IgG binding proteins and IgG,

(d) polymeric chelators and chelat s,

(e) complementary nucleic acids.

Affinity members also include entities participating in catalytic reactions, for instance enzymes, enzyme substrates, cofactors, cosubstrates etc. Members of cell-cell and cell-surface interactions and a synthetic mimetics of bioproduced affinity members are also included. The term ligand also includes more or less complex organic molecules that binds through affinity to complex biomolecules, for instance having oligo or polypeptide structure (including proteins), oligo and polynucleotide structure (including nucleic acids), oligo- or polysaccharide structures etc.

Further examples of ligands to be used in the present invention include dyes such as CIBACRONE BLUE F3G-A™ (manufactured by Fluka) and other complex dyes, iminodiacetic acid, sugar chains such as glucose, proteins such as heparin and lectin, biotin, benzamidine, lysine, arginine, peptides and DNA. It is also according to the invention possible to control the bonding ability of the target substance by covalently bonding the affinitive substance of the target substance to the support via a spacer such as a bivalent alkyl group or an ethylene oxide group.

The base matrix (e.g. chromatographic packings)

The separation medium to be used in the inventive method comprises a base matrix (carrier) which may be based on organic and/or inorganic material. In case the liquid used is aqueous, the base matrix is preferably hydrophilic. This in particular appli s to targ t substances that are biomolecules of the kind discuss d above.

The base matrix is preferably based on a polymer, which preferably is insoluble and more or less swellable in wat r, preferably to a gel. Hydrophobic polymers that have be n derivatized to become hydrophilic are included in this definition. Suitable polymers are polyhydroxy polymers, e.g. based on polysaccharides, such as agarose, dextran, cellulose, starch, pullulan, etc. and completely synthetic polymers, such as polyacrylic acid amide, polymethacrylic acid amide, poly(hydroxyalkylvinyl ethers), poly(hydroxyalkylacrylates) and polymethacrylates (e.g. polyglycidylmethacrylate), polyvinylalcohols and polymers based on styrenes and divinylbenzenes, and copolymers in which two or more of the monomers corresponding to the above-mentioned polymers are included. Polymers, which are soluble in water, may be derivatized to become insoluble, e.g. by cross-linking and by coupling to an insoluble body via adsorption or covalent binding. Hydrophilic groups can be introduced on hydrophobic polymers (e.g. on copolymers of monovinyl and divinylbenzenes) by polymerization of monomers exhibiting groups which can be converted to OH, or by hydrophilization of the final polymer, e.g. by adsorption of suitable compounds, such as hydrophilic polymers.

Suitable inorganic materials to be used in base matrices are silica, zirconium oxide, graphite, tantalum oxide etc.

Preferred base matrices lack groups that are unstable against hyrolysis, such as silan, ester, amide groups and groups present in silica as such. Preferred base matrices contain functional groups that can be used for attaching covalently the stimulus-responsive polymer and/or the ligand. This kind of

functional groups are illustrated by hydroxy, carboxy, amino groups etc.

The matrix may be porous or non-porous. This means that the matrix may be fully or partially permeable (porous) or completely impermeable to the compound to be removed (non-porous).

The pores may have sizes ≥ 0.1 μm, such as ≥ 0.5 μm, by which is meant that a sphere ≥ 0.1 μm respective ≥ 0.5 μm in diameter is able to pass through. An applied liquid may be able to flow through this kind of pore system (convective pore system). In case the support matrix is in form of beads packed to a bed, the ratio between the pore sizes of the convective pore system and the diameter of the particles typically is in the interval 0.01-0.3, with preference for 0.05-0.2. Pores having sizes ≥ 0.1 μm, such as ≥ 0.5 μm, are often called macropores.

The base matrix may also have pores with sizes ≤ 0.5 μm, such as ≤ 0.1 μm by which is meant that only spheres with diameters ≤ 0.5 μm, such as ≤ 0.1 μm, can pass through. Pores having sizes ≤ 0.5 μm, such as ≤ 0.1 μm, are often called micropores.

In one embodiment of the invention, the base matrix is in the form of irregular or spherical particles with sizes in the range of 1-1000 μm, preferably 5-50 μm for high performance applications and 50-300 μm for preparative purposes. Particles to be used may be monodisperse (monosized) or polydispersed (polysized). By the term monodispersed particles is meant a particle populations having more than 95% of the particles with sizes within their mean diameter ± 5%, which in the context of the pres nt invention contemplate the expression particles of

uniform size. Polydispersed particles encompass other populations of particles.

The base matrix may also be in form of a monolith having at least macropores as defined above. Alternative geometric forms are the interior walls of tubes and the like

The stimulus responsive polymer and the ligand as defined above may be attached to the outer surfaces and/or on the interior surfaces (macropore and/or micropore surfaces) of the base matrix. As discussed above the stimulus responsive polymer and the ligand may be attached to the base matrix by physical adsorption and/or covalent attachment, preferably the latter.

It is particularly preferable to use as the support/base matrix hydrophilic porous polymer particles having a uniform particle size, which are produced by the membrane emulsification method followed by a chemical treatment with an acidic substance or a basic substance as discussed above.

The membrane emulsification method as used in the present invention is a method which comprises passing a first liquid through a glass membrane, preferably made of glass, into a second liquid which is mot miscible with the first liquid, thus forming droplets of an essentially size in the second liquid. This method is described in, for example, S. Omi, K. Katami, A. Yamamoto and M. Iso. J. Appl. Polym. Sci., 51 (1994) 1-11. In case the first liquid contains a polymerizable monomer and the droplets are subjected to polymerization, particles will form in the second liquid.

Thus the preferr d the support materials (base matrices) according to the inventor's novel finding is produc d in the following manner: A liquid mixture (first liquid) is prepared

from a monomer, which serves as the starting material for polymer particles, and a diluent, etc. Next, one side of a porous glass membran is filled with this liquid mixture and the opposite side with a second liquid which is not miscible with the first liquid. Pressure is the applied to the liquid mixture so that it passes through the membrane and forms droplets in the second liquid. For instance the first liquid may be immiscible with water and the second liquid aqueous containing an emulsion stabilizer, etc. to give an emulsion consisting of droplets of an essentially uniform size. Subsequently, polymerization is carried out by, for example, heating to thereby give latex particles having a uniform particle size. Provided that the monomer contains a group reactive with an acidic or basic substance, the latex particles can be stirred in a solution containing this kind of substances to give hydrophilic porous polymer particles. During this post-treatment, reactive functional groups such as amino groups can be introduced into the support. By using these reactive functional groups, the stimulus-responsive polymer or the ligand can be covalently attached to the support.

Examples of the monomer to be used in producing the hydrophilic porous polymer particles having a uniform particle size include glycidyl acrylate, glycidyl methacrylate, diacrylates (for example, ethylene diacrylate), dimethacrylates (for example, ethylene dimethacrylate), glycidyl vinylbenzyl ether and divinylbenzene. It is also possible to combine these monomers.

The diluent to be used in the production of the hydrophilic porous polymer particles having a uniform particle size may be an arbitrary compound as long as it is not polymerizabl with

the monomers us d. Examples thereof include aromatic solvents/compounds, such as toluene and aliphatic compounds such as dodecane.

Examples of the acidic substance or basic substance to be used in the production of the hydrophilic porous polymer particles having a uniform particle size include sulfuric acid, hydrochloric acid, nitric acid, acetic acid, sodium hydroxide, ammonia and aliphatic diamines such as 1,6-diaminohexane.

Manufacture of the separation material of the present invention

The affinity-controlling material/separation material according to the present invention can be produced by, for example,

(a) a method which comprises covalently attaching a stimulus-responsive polymer or a copolymer thereof to a support and then covalently attaching an affinitive substance of a target substance to the support; or

(b) a method which comprises covalently attaching the affinitive substance of the target substance to the support and then covalently attaching the stimulus-responsive polymer or a copolymer thereof to the support; or

(c) a method which comprises covalently attaching the affinitive substance of the target substance and the stimulus-responsive polymer or a copolymer thereof respectively to the support at the same time.

Examples

The present invention is illustrated below in more d tail with reference to the following examples, but is not to be

construed as being limited thereto.

Example 1

1. Synthesis of stimulation-responsive polymer

N-isopropylacrylamide (20 g), 3-mercaptopropionic acid (0.18 g), and 2,2'-azobis(4-cyanovaleric acid) (0.27g) were dissolved in tetrahydrofuran (200 ml). The resulting solution was placed in a polymerization tube. Oxygen was removed from the solution by the freezing and thawing deaeration method. The polymerization was performed at 60°C for 2 hours. Poly(N-isopropylacrylamide) having a carboxyl group at one end of its molecule was reprecipitated using diethyl ether as solvent.

The molecular weight of the obtained polymer was determined by gel permeation chromatography (GPC) and end-group analysis. GPC was performed using dimethylformamide containing 10 mM lithium bromide as a mobile phase, a column á-3000 (TOSOH Co., Japan) column, and polystyrene as a standard reference material. The number average molecular weight and the weight average molecular weight of the synthesized poly(N-isopropylacrylamide) were found to be about 4,500 and 10,000, respectively. The carboxyl terminal groups of the synthesized poly(N-isopropylacrylamide) were determined by end group analysis with a 0.01N sodium hydroxide solution. As a result, the number average molecular weight was about 5000. It was thus confirmed that the number average molecular weight of the synthesized poly(N-isopropylacrylamide) determined by GPC is essentially the same as that determined by end group analysis.

The synthesized poly(N-isopropylacrylamide)(10 g), N-hydroxysuccinimide (0.25 g), and N,N'-dicyclohexyl-

carbodiimide (0.45 g) were dissolved in tetrahydrofuran (60 ml), and the resulting solution was stirred at room temperature for 12 hours. The resulting precipitate was collected by filtration and reprecipitated in diethyl ether to give poly(N-isopropylacrylamide) whose carboxyl group at one end is esterified with N-hydroxysuccinimide.

2. Synthesis of hydrophilic porous polymer particles with a uniform particle size

The starting materials, glycidyl methacrylate (3.1 ml), ethylene dimethacrylate (1.9 ml), toluene (7.1 ml), dodecane (0.4 ml), and 2,2'-Azobis(2,4-dimethyl-valeronitrile) 50 mg were passed through an MPG (Micro Porous Glass) pipe with the average pore size of 1.95 μm under pressure and extruded into a 2 wt% polyvinyl alcohol solution to prepare a O/W emulsion. The emulsion was subjected to polymerization at 70°C for 6 hours, and the latex particles with a uniform particle size were in a high yield. The average particle diameter of the latex particles was 12.5μm, the CV (coefficient of variation)value was 12.4%, and the particles were uniform in size. The synthesized latex particles (3.5 g) were dispersed into an aqueous solution (160 ml) containing 1,6-hexyldiamine (1.8 g), and the mixture was stirred at 30°C for 2 hours.

The hydrochloric acid-calcium chloride method (Nakamura et al., Kobunshi Ronbunshu, 38(7) (1981) 485-491.) gave that the latex particles had 3.1 mmol/g of epoxy groups on their surfaces prior to the treatment with 1,6-hexyldiamine. Furthermore, the assay using titration revealed that 0.36 mmol/g of amino groups were introduced onto the surfaces of the hydrophilic porous

polymer particles by the treatment with 1,6-hexyldiamine.

The 1,6-hexyldiamine-treated hydrophilic porous polymer particles (3.5 g) were then added to 10 ml of acetic anhydride, the solution was stirred to acetylate the amino groups, thereby obtaining amidated particles. These amidated particles did not adsorb bovine serum albumin (BSA) in a 20 mM phosphate buffer (pH 7.0) used as a mobile phase. This indicates that the amidated hydrophilic porous polymer particles had a hydrophilicity sufficient to render them suitable as a chromatography carrier (base matrix) protein separation by affinity chromatography.

3. Immobilization of poly(N-isopropylacrylamide) on the support

A mixture containing 4.5 g of the 1,6-hexyldiamine-treated hydrophilic porous polymer particles from the previous partobtained above, 4.5 g of poly(N-isopropylacrylamide) whose carboxyl group at one end of its molecule is esterified with N-hydroxysuccinimide (from part 1 of this example), and 75 ml of acetonitrile was stirred at room temperature for 12 hours. The particles were then washed with acetonitrile, tetrahydrofuran, methanol, and acetone, and dried at room temperature. Elementary analysis revealed that 3.4 wt% of poly(N-isopropylacrylamide) was immobilized on the particles. In addition, to assess the temperature-dependent affinity-controlling capabilities, the packing material (noCB) was prepared by acetylating the remaining amino groups in the support with acetic anhydride.

4. Imm bilization of Cibacron Blue F3G-A on the supp rt

A mixture of the poly(N-isopropylacrylamide)-

immobilized support (0.70 g) (from part 3 of this example), either of 1,3-butadiene epoxide (0.09 ml) or ethylene glycol diglycidyl ether (0.21g), and acetonitrile (10 ml) was stirred at 30°C for 1 hour to allow residual amino groups in the support to react with one of the epoxy groups in the diepoxide compound that served as a spacer. The unreacted amino groups of the support were acetylated by adding acetic anhydride (0.11 ml) to the suspension followed by stirring at 30°C for 1 hour. The resulting support on which the spacer and poly(N-isopropylacrylamide) are immobilized was washed with acetonitrile and acetone, and then dried at room temperature.

A mixture of the support on which poly(N-isopropyl-acrylamide) and spacer are immobilized (0.61 g), aminohexylated Cibacron Blue F3G-A (0.89 g), and water (10 ml) was adjusted to pH 11 with sodium hydroxide and stirred at 25°C for 3 hours to prepare the packing material that is the support on which poly(N-isopropylacrylamide) and Cibacron Blue F3G-A are immobilized. The amount of immobilized Cibacron Blue F3G-A was determined by titration. The packing material (CB-4) prepared using 1,3-butadiene epoxide as a spacer contained 21 μmol/g of Cibacron Blue F3G-A, whereas the packing material (CB-10) prepared using ethylene glycol diglycidyl ether as a spacer 12 μmol/g.

<u>Example 2</u>

1. Filling of the packing materials

Each of the packing materials, noCB, CB-4, and CB-10, was packed in a stainless-st el column of 4.6 mm in inner diam ter and 30 mm in length by the wet packing method using water.

2. Assay for the amount of BSA adsorbed by the packing material

The amounts of BSA adsorbed by the respective packing materials were determined at 40°C using a citrate buffer with pH 5 (I=0.01) as a mobile phase and calculated based on the breakthrough curves taking the result obtained at 20°C as a standard. The results are shown in Table 1.

Table 1

Packing material	Amount of adsorbed BSA per gram packing material
noCB	6.7 μg
CB-4	23.4 μg
CB-10	73.8 μg

CB-10 adsorbed more BSA than CB-4, indicating that the length of the spacer between the support and Cibacron Blue F3G-A influences the BSA adsorption. The result also suggested that poly(N-isopropylacrylamide) immobilized on the support does not significantly influence the BSA adsorption.

3. Temperature-dependent affinity control of the packing materials in affinity chromatography

BSA was allowed to be adsorbed at 40°C by Cibacron Blue F3G-A, the ligand of the packing material CB-10. The temperature was then shifted down to 20°C to change the structur of the

stimulus-responsive polymer. It was confirmed that BSA was released from the packing material and eluted in the mobile phase due to the structural change of the polymer. The result is shown in Fig. 1. BSA (111 μg) was loaded onto a column of CB-10 at 40°C using a citrate buffer with pH 5 (I = 0.01) as a mobile phase. The amount of BSA in the eluate was measured by using MICRO BCA™ PROTEIN ASSAY REAGENT KIT (manufactured by Pierce). An excess amount of BSA was eluted in the first 1 to 4 ml aliquot of the eluate. The mobile phase was passed through the column at 40°C until the eluate volume reached 6 ml, confirming that no more BSA is eluted. The flow of the mobile phase was then stopped and the column was cooled at 20°C for 20 minutes. When the mobile phase flow was resumed at 20°C, the BSA adsorbed to the ligand at 40°C was released and eluted from the column (7 to 9 ml of the eluate), which resulted from the structural change of the poly(N-isopropylacrylamide) immobilized on the support. Moreover, the amount of BSA eluted in a temperature-dependent manner was 90% of the total amount of BSA adsorbed by CB-10. These results revealed that a target substance can be removed or separated and purified from a solution using a material comprising a support/base matrix to which a stimulus-responsive polymer and a ligand having affinity for the target substance are covalently attached via separate links. The results also show that the affinity between the target substance and the ligand can be controlled by physical stimulus such as temperature.

Industrial Applicability

The affinity-controlling material according to the

present invention is advantageous in the following points.

1) Since no chemically severe condition is needed in the separation and purification of a target substance, the activity or recovery yield of a physiologically active substance, etc. can be largely elevated compared with the conventional separation/purification methods.

2) Owing to the covalent bonds of the affinitive substance of the target substance and the stimulus-responsive polymer to the support, it is not feared that they might peel off and disturb the separation/purification.

3) When the affinity-controlling material of the present invention is used as an affinity chromatographic packing, the packing can be quickly regenerated compared with the conventional supports.

4) The affinity-controlling material of the present invention makes it possible to separate and purify various types of target substances, which cannot be achieved by the conventional affinity chromatographic packings.